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Postglacial Lineage Admixture in the Contact Zones of the Two Japanese Deciduous Broad-leaved Tree Species Estimated by Nuclear Microsatellite and Chloroplast DNA Markers

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When historically isolated populations meet during postglacial expansion, a mixed distribution of distinct DNA lineages called contact zones is created. The gradual dissolution of the spatial genetic structures in contact zones should be related to differences in pollen and seed dispersal, given no restriction on gene flow by e.g. reproductive isolation. We aimed to clarify effects of pollen dispersal modes on nuclear DNA (nrDNA) genetic structures of two codistributed species with different pollen dispersal modes, by analyzing nuclear microsatellites of the insect-pollinated *Magnolia obovata* and the wind-pollinated *Carpinus laxiflora,* which show highly consistent contact zone locations in terms of chloroplast DNA (cpDNA). The genetic structure based on the nrDNA and that based on the cpDNA were concordant in *M. obovata*, but not so concordant in *C. laxiflora*. Pollen dispersal ability is higher in the wind-pollinated *C. laxiflora* than in *M. obovata*, resulting in the higher estimated pollen/seed migration ratio in *C. laxiflora* than in *M. obovata*. Therefore, the extent of postglacial lineage admixture in nrDNA was predominant in *C. laxiflora*. Our results suggested that differences in pollen dispersal ability may affect the nrDNA genetic structure between co-distributed species with common migration histories in the same area.

Key words: admixture, contact zone, deciduous broad-leaved forest, gene flow, microsatellites, phylogeographic studies

Climate oscillations during the Quaternary affected geographical distribution of various plant and animal species (Hewitt 2004). During climatic oscillations, the ranges of organisms might have occasionally been divided into several isolated populations. The genetic structure in extant populations may be the result of such historical changes in distribution (Hewitt 2000). Several organisms exhibit intraspecific genetic structures consistent with expected patterns from historical distributional changes suggested by paleontological studies (King & Ferris 1998, Abbott *et al.* 2000, Palme & Vendramin 2002, Petit *et al.* 2002, McLachlan *et al.* 2005, Magri *et al.* 2006). Contact zones are defined as areas where isolated populations from different refugia meet during postglacial expansion and distinct DNA lineages are consequently able to mix (Harrison 1993, Taberlet *et al.* 1998). Although secondary contacts of isolated lineages may result in other evolutionary consequences, such as reinforcement (Song *et al.* 2009, Lee & Mitchell-Olds 2011, 2013), the genetic structure of once separate populations in such contact zones are expected to gradually dissolve through postglacial mixing as long as gene flow between the lineages is not

strongly restricted by reproductive isolation or other factors.

In plants, the rate of genetic mixing may be related to differences in dispersability of pollen and seeds. In particular, it is easy to expect that species with different modes of pollen dispersal would differ in dispersability. Based on data from studies using allozyme markers, Hamrick *et al.* (1990) reported that wind-pollinated plant species tend to show lower G_{ST} values than animal-pollinated species. In other words, they suggested that immigration rates and gene flow in wind-pollinated species are usually greater than in animal-pollinated species.

To examine the influence of different pollen dispersal modes on genetic structure, analyses using bi-parentally inherited nuclear genetic markers are indispensable because maternally inherited chloroplast DNA (cpDNA) markers can migrate only through seeds, and thus are a reflection of seed dispersal. In contrast, nuclear DNA (nrDNA) markers migrate through both pollen and seeds. Thus, it cannot be determined whether pollen or seed dispersal is more influential when analyses are based only on nrDNA or cpDNA markers.

Recently, several phylogeographic studies of plants have used both nuclear and cytoplasmic DNA markers (Bai *et al.* 2010, Sakaguchi *et al.* 2012, Qi *et al.* 2012, Ohtani *et al.* 2013, Bai *et al.* 2014). For example, in wind-pollinated *Juglans mandshurica* Maxim. and *J. cathayensis* Dode, the genetic structure observed on the basis of nrDNA SSR markers was not as informative as studies based on cpDNA markers (Bai *et al.* 2010, 2014). In contrast, in insect-pollinated *Kalopanax septemlobus* (Thunb.) Koidz. and *Shorea leprosula* Miq., similar genetic structures were observed based on both nrDNA and cpDNA markers (Sakaguchi *et al.* 2012, Ohtani *et al.* 2013). Those results suggest that differences in pollen dispersability between wind- and insect-pollinated species are significantly responsible for differences between cpDNA and nrDNA genetic structures.

The previous studies, however, did not compare the genetic structure between codistributed species with different pollen dispersal modes in the same area. Therefore, the effects of dispersal mode on genetic structures remains unclear. We aimed to clarify the effects of pollen dispersal mode on the nrDNA genetic structure of several codistributed species that have different dispersal modes but similar distribution ranges and similar cpDNA genetic structures.

Four deciduous broad-leaved tree species in western Honshu, Japan, *Carpinus laxiflora* (Siebold & Zucc.) Blume, *C. tschonoskii* Maxim., *C. japonica* Blume, and *Magnolia obovata* Thunb., with various seed and/or pollen dispersal modes, have been reported to show similar east–west genetic differentiation patterns in their cpDNAs (Iwasaki *et al.* 2010, 2012). Furthermore, Tono *et al.* (2015) reported that the contact zones of the four species were highly consistent with each other in terms of their cpDNAs. These observations indicate that the migration histories and extent of postglacial genetic admixture through seeds were similar among the four species. These species therefore provide us with a suitable system for examining the influence of different pollen dispersal modes on genetic structure.

In this study, we focused on two species, the insect-pollinated *Magnolia obovata* and the wind-pollinated *Carpinus laxiflora*, which are a subset of the four species examined in the previous study based only on cpDNA markers (Tono *et al.* 2015). To examine the influence of different pollen dispersal modes on genetic structure, we compared the geographic patterns of genetic differentiation based on nuclear microsatellite and cpDNA markers between the insect-pollinated *M. obovata* and the wind-pollinated *C. laxiflora*. We addressed the following two questions: (1) Are the geographic patterns of genetic differentiation based on nrDNA markers similar to those based on cpDNA markers? (2) Are the geographic patterns of genetic differentiation based on nrDNA markers different between the two species with different pollen dispersal modes?

Materials and Methods

Sampling of the plants

We used silica gel to dry leaf samples from 250 and 264 individuals of *Magnolia obovata* and *Carpinus laxiflora* from 45 and 43 populations, respectively, in the Kinki-Chugoku region (Appendix 1 and Fig. 1). Of these, 224 and 232 samples from 42 and 38 populations, respectively, had already been examined in a previous study (Tono *et al.* 2015). Thus, 26 and 32 individuals from 15 and 10 populations, respectively, were newly sampled in this study. Voucher specimens have been deposited in the Makino Herbarium of Tokyo Metropolitan University (MAK).

cpDNA sequencing and haplotype classification

Twenty-six samples of *Magnolia obovata* and 32 samples of *Carpinus laxiflora* were newly analyzed in this study. In addition, the cpDNA haplotype data from the two species were cited by Tono *et al.* (2015). The methods for DNA extraction, polymerase chain reaction (PCR) amplification of cpDNA noncoding regions, sequencing, and classification of the eastern and western haplotypes followed those of Tono *et al.* (2015). The primers used for amplifying and sequencing the cpDNA non-coding regions are shown in Appendix 2. In total, cpDNA haplotypes were determined for 250 and 264 individuals of *M. obovata* and *C. laxiflora* from 45 and 43 populations, respectively.

Development of microsatellite markers for Carpinus laxiflora

Microsatellite markers were developed through pyrosequencing with enriched DNA libraries. Total DNA was extracted from the leaf samples of *Carpinus laxiflora* (voucher: AT3057) using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The DNA was shotgun sequenced using the Roche 454 GS Junior using a GS Junior Titanium Sequencing Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions on quarter plates with libraries of three other species identified by molecular identifier (MID) tags. From the obtained sequences, candidates of microsatellite loci were screened using MSATCOMMANDER (Faircloth 2008). The sequences with either ≥8 dinucleotide repeats, ≥8 trinucleotide repeats, ≥6 tetra nucleotide repeats, ≥6 pentanucleotide repeats, or ≥6 hexanucleotide repeats were searched using Primer3 program (Rozen & Skaletsky 2000) embedded in MSATCOMMANDER. For all loci, a 19-bp M13 tail (5'-CACGACGTTGTAAAACGAC-3') was added to the 5' end as the forward primer sequence following the method of Schuelke (2000).

PCR amplification tests were performed for eight individuals from six populations in a final volume of 5 μL using the standard protocol of the QIAGEN Multiplex PCR Kit (QIAGEN) for single-plex PCR (one primer pair per reaction). Each reaction contained 0.2 μM reverse primer, 0.1 μM FAM-labeled M13 primer, and 0.1 μM forward primer. PCR amplification included initial denaturation at 95°C for 15 min; 30 cycles of reactions at 94°C for 0.5 min, 57°C for 1.5 min, and 72°C for 1 min; and a final extension at 60°C for 30 min. PCR products were analyzed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). PCR product sizes were determined by comparison with GeneScan 500 or 600 LIZ Size Standard (Applied Biosystems) using GeneScan analysis (Applied Biosystems). The results were analyzed using GeneMapper version 4.0 (Applied Biosystems).

The successfully amplified loci were analyzed for 31 individuals of *Carpinus laxiflora* from one population (Gonami Pass, Ooi, Fukui Prefecture, Japan) with the same protocol used in the amplification test described above. For each locus, the number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E), and fixation index (F_{IS}) were calculated using GenAlEx version 6.5 (Peakall & Smouse 2006). The Hardy-Weinberg equilibrium (HWE) was tested for each locus using GenAlEx version 6.5 (Peakall & Smouse 2006) with Bonferroni corrections. The linkage disequilibriums (LDs) between loci were examined using exact tests in FSTAT version 2.9.3.2 (Goudet 2002) with Bonferroni cor-

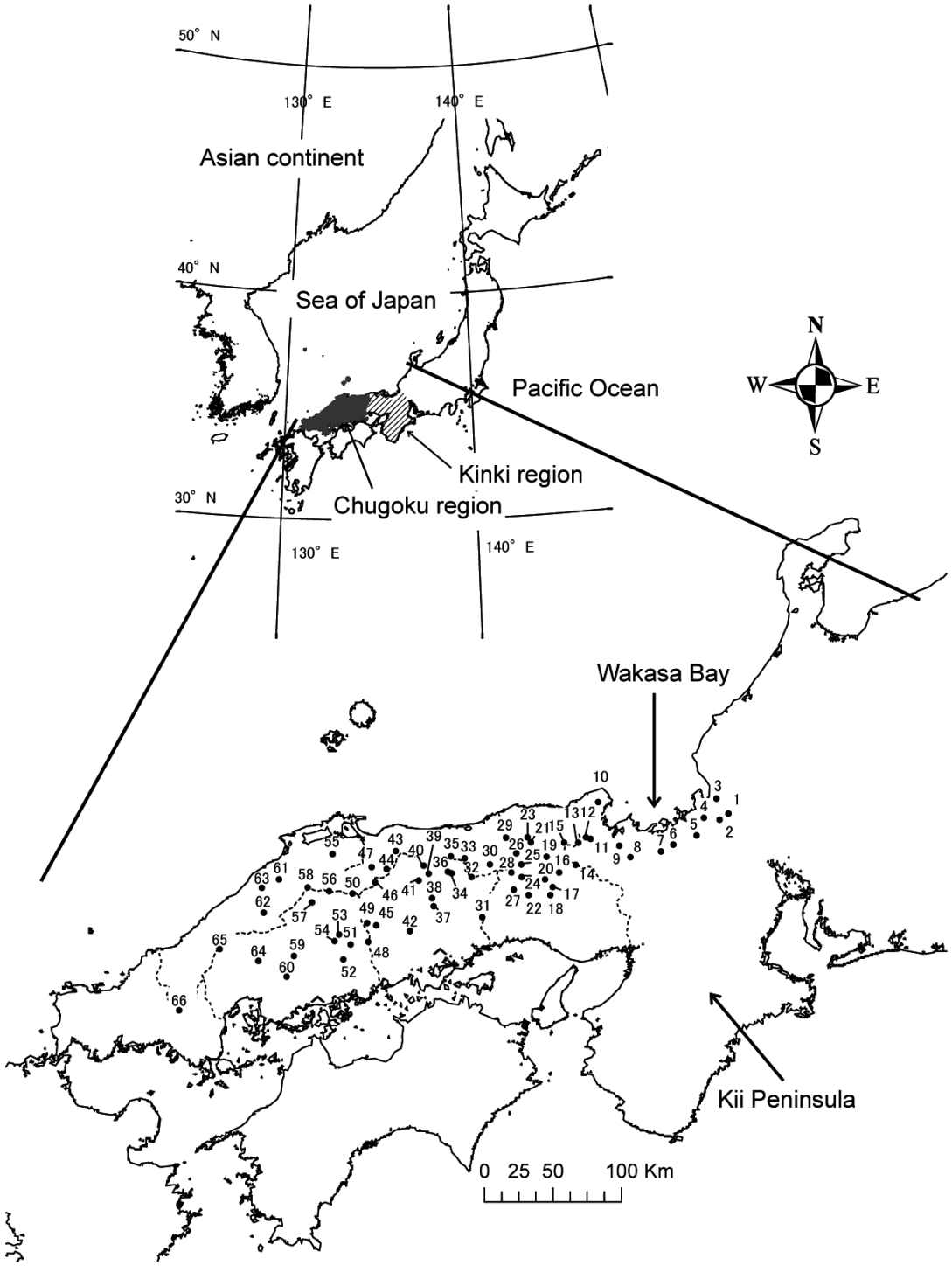

FIG. 1. Sampling sites in the Kinki-Chugoku region. Detailed information for each site is shown in Appendix 1.

rections.

Microsatellite genotyping

For *Magnolia obovata*, seven microsatellite markers developed by Isagi *et al.* (1999) (M10D6, M6D3, M6D4, M15D5, M10D3, M6D10, and M17D5) were used to determine the microsatellite genotypes of 250 individuals from 45 populations. The individuals were obtained from the same samples that were used in the cpDNA analyses. For *Carpinus laxiflora*, 11 microsatellite markers developed in the present study (Carp2, Carp3, Carp6, Carp11, Carp13, Carp16, Carp17, Carp26, Carp27, Carp34, and Carp41) were used to determine the microsatellite genotypes of 264 individuals from 43 populations. These individuals were also obtained from the same samples used in the cpDNA analyses. Primer information is shown in Appendix 3 and 4. For each primer set, the forward primer was synthesized with a tag sequence (FAM = 5'-CACGACGTTGTAAAACGAC-3'; NED = 5'-CTATAGGGCACGCGTGGT-3' VIC = 5'-TGTGGAATTGTGAGCGG-3') added to its 5' end using the method of Schuelke (2000). PCR amplification was performed with a final volume of 5 μL using the standard protocol of the QIAGEN Multiplex PCR Kit (QIAGEN) with two- or three-plex PCR (two or three primer pairs per reaction). Three primers were used in each reaction: 0.066 μM reverse primer, 0.033 μM fluorescently labeled primer, and 0.033 μM forward primer. The methods for PCR amplification and determination of the product size were the same as those for microsatellite marker development, except for different annealing temperatures (50–57°C).

Microsatellite data analyses

To check whether each locus met the requirements for neutral evolution, population genetic analysis was performed. At first, neutral evolution of the analyzed microsatellite markers was tested for both species using the F_{ST}-outlier approach (Beaumont & Nichols 1996, Beaumont 2005) implemented in LOSITAN (Antao *et al.* 2008). In this analysis, the relationship between the expected heterozygosity (H_E) and Wright's coefficient of genetic differentiation (F_{ST}) was examined to identify outlier loci with excessively high or low F_{ST} values compared with the neutral expectation under an island migration model. This test was performed using an infinite allele model with 50,000 simulations, and a confidence interval of 0.95. LOSITAN analysis was performed for each population that contained more than 15 individuals. Second, for *Magnolia obovata*, we tested for departure from HWE at each locus for each population that contained more than nine individuals using an exact test in GenAlEx version 6.5 (Peakall & Smouse 2006) with Bonferroni corrections. LD between loci was also tested using an exact test in FSTAT version 2.9.3.2 (Goudet 2002). Bonferroni corrections were applied to all multiple statistical tests.

To evaluate genetic differentiation in the two species of trees, G_{ST} (Nei 1987) was calculated using FSTAT version 2.9.3.2 (Goudet 2002). In addition, G'_{ST} and standardized values of G_{ST} were calculated manually (Hedrick 2005). To elucidate the characteristics of each population that contained more than four individuals, the following parameters were calculated on the basis of the multilocus genotype data using GenAlEx version 6.5 (Peakall & Smouse 2006) and FSTAT version 2.9.3.2: allelic richness (*AR*), the total number of private alleles (*PA*), observed heterozygosity (H_O), and expected heterozygosity (H_E). The values for *AR* and *PA* were calculated by rarefying to five individuals using FSTAT version 2.9.3.2 and HP-RARE version 1.1 (Kalinowski 2005), respectively.

STRUCTURE analysis

The intraspecific genetic structure of the two species was estimated by Bayesian clustering using STRUCTURE version 2.3.4 (Pritchard *et al.* 2000, Falush *et al.* 2003, 2007). This analysis can reveal the population structure with *a priori* assignment of individuals to populations on the basis of multilocus genotype data. The assignment probabilities of clusters were estimated using the Markov Chain Monte Carlo (MCMC) method, assuming gene pools with the least possible LD and smallest possible departure from HWE. The

admixture model and LOCPRIOR model with correlated allele frequencies were used (Falush *et al.* 2003, Hubisz *et al.* 2009). Ten independent runs were performed using a burn-in period of 100,000 and posterior probabilities were obtained from 100,000 iterations for each number of clusters, K = 1 to 10. The most likely value of K was assessed using the ΔK values (Evanno *et al.* 2005).

Pollen/seed migration ratios

To estimate the relative rates of pollen and seed migration among populations, the pollen/seed migration ratio (r) was calculated using the following equation: (pollen flow/seed flow) = $\{(1/G'_{STn} - 1) - 2\,(1/G'_{STc} - 1)\}/(1/G'_{STc} - 1)$, where G'_{STc} is the G'_{ST} of cpDNA and G'_{STn} is the G'_{ST} of nrDNA. This is a modification of the equation by Ennos (1994) with the substitution of G_{ST} values

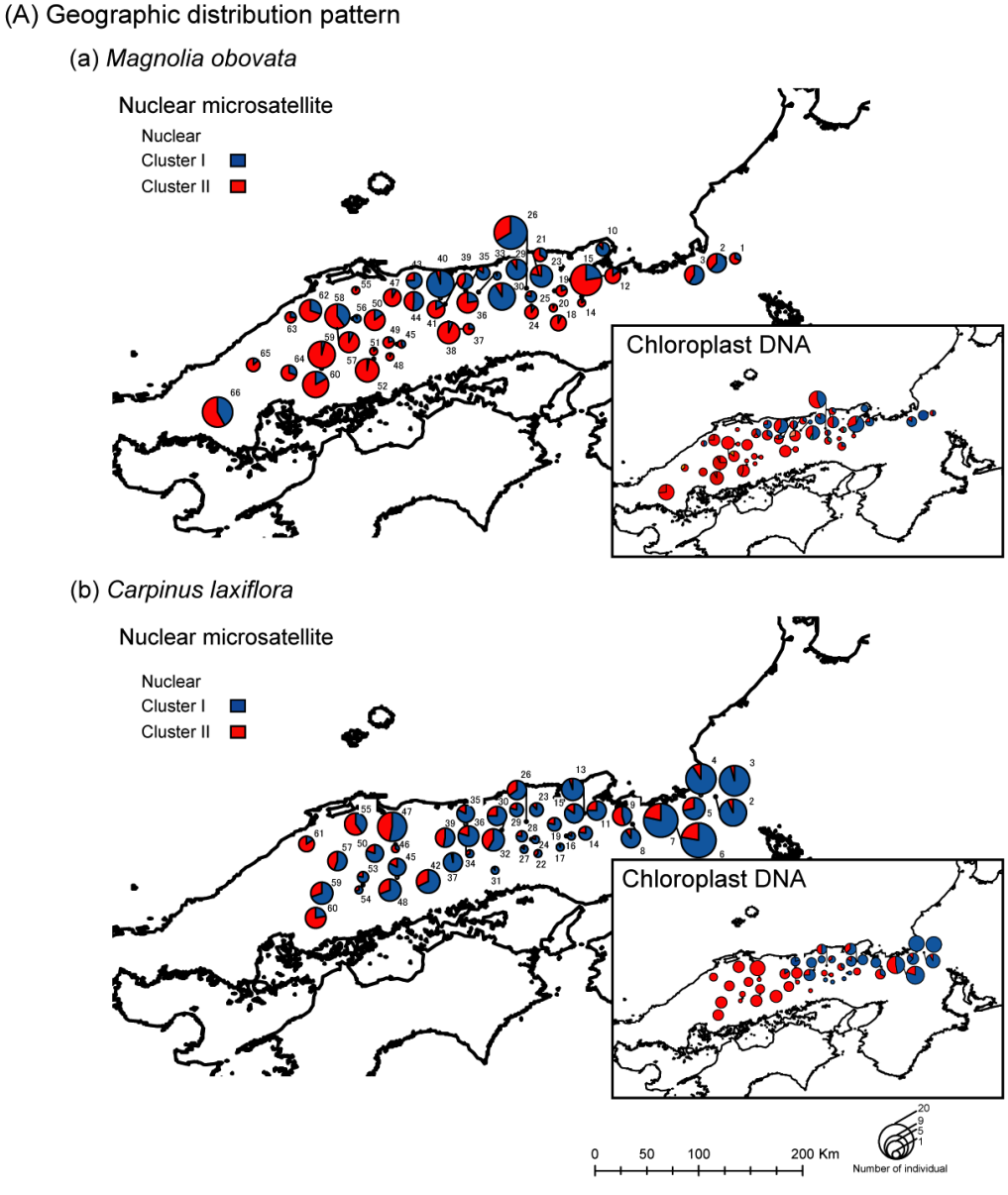

FIG. 2. (A) Geographic distribution pattern of two clusters estimated by STRUCTURE analysis on basis of nuclear microsatellite data and composition of chloroplast DNA haplotypes for *Magnolia obovata* (a) and *Carpinus laxiflora* (b). For nuclear microsatellite data, blue represents Cluster I; red represents Cluster II. For chloroplast DNA, eastern and western haplotypes are in blue and red, respectively. Number of samples analyzed per population and haplotype composition is proportional to circle size. Population numbers correspond to those in Appendix 1.

for F_{ST} values. The values of G_{ST} and G'_{ST} (standardized values of G_{ST}; Hedrick 2005) of cpDNA were calculated by PERMUT version 2.0 (http://www.pierroton.inra.fr/genetics/labo/Software/) (Pons & Petit 1996) and manually, respectively.

Results

Geographic distribution patterns of cpDNA haplotypes

In *Magnolia obovata*, five haplotypes (A, B, C, F, and G) were found. Three (A, C, and F) and two haplotypes (B and G) were classified as eastern and western haplotypes, respectively, according to Tono *et al.* (2015). In *Carpinus laxiflora*, two haplotypes (A and B) were found. Haplotype B and A were classified as eastern and western haplotypes, respectively, according to Tono *et al.* (2015). All of the haplotypes observed in the two species had already been reported in Tono *et al.* (2015). The geographic distribution patterns of the eastern and western cpDNA haplotypes in the two species are shown in Fig. 2. After combining the newly obtained data from this study with data cited in Tono *et al.* (2015), proportions of the eastern and western haplotypes still differed between the eastern and western parts of the Kinki-Chugoku region. The general tendencies were the same as in previous studies (Iwasaki *et al.* 2012, Tono *et al.* 2015).

Microsatellite marker development for Carpinus laxiflora

The *de novo* pyrosequencing produced 15,602 reads with an average length of 435.62 bp. Three hundred and sixty three sequences with simple sequence repeats were found by MSATCOMMANDER. Primer sets were successfully designed for a total of 41 microsatellite loci. Of these, 14 primer pairs (Carp2, Carp3, Carp6, Carp11, Carp13, Carp16, Carp17, Carp20, Carp21 Carp24, Carp26, Carp27, Carp34, and Carp41) successfully amplified DNA fragments through PCR (Appendix 4). The remaining 27 primer pairs failed to amplify DNA fragments with the expected product size. Characteristics of these 14 microsatellite markers are shown in Appendix 5. Only one marker (Carp21) showed a significant deviation from HWE for the test population ($P < 0.05$ after Bonferroni correction). Significant LD was not detected for any pairs of loci. It was difficult to score fragment sizes for two markers

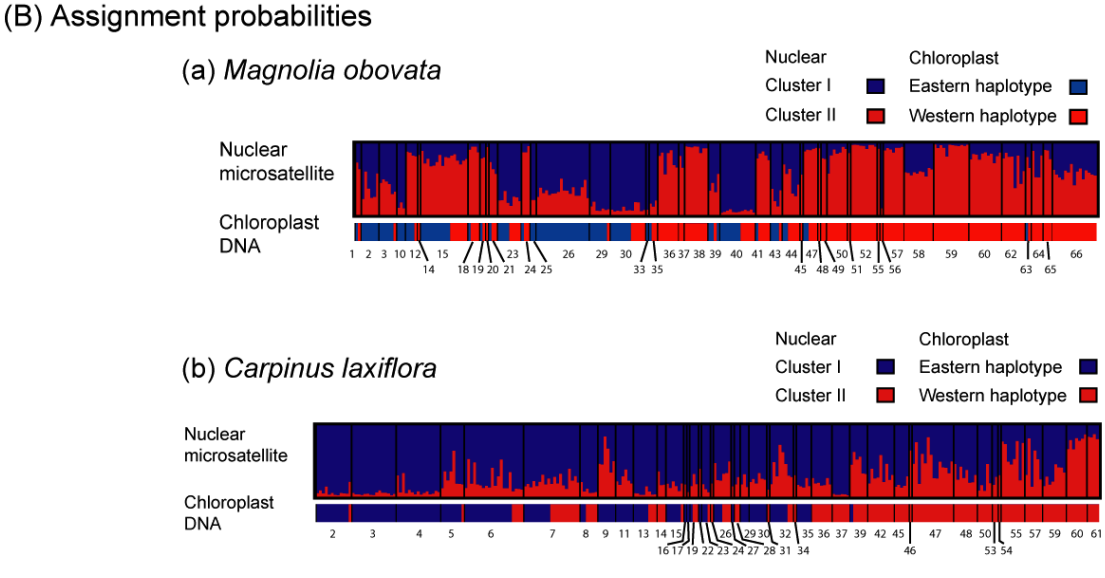

FIG. 2. (B) Assignment probabilities into two STRUCTURE clusters (top) and cpDNA haplotypes (bottom) for each individual. Blue represents proportion of cluster I; red represents cluster II. In chloroplast DNA results, blue represents eastern haplotypes; red represents western haplotypes. Numbers at bottom represent population numbers. Populations are sorted from east to west.

TABLE 1. Parameters of genetic differentiation of chloroplasts, nuclear DNA polymorphisms, and pollen/seed migration ratios.

	Magnolia obovata	*Carpinus laxiflora*
Chloroplasts G_{ST} (G_{STc})	0.400	0.675
Chloroplasts G'_{ST} (G'_{STc})	0.675	0.827
Nuclear G_{ST} (G_{STn})	0.044	0.029
Nuclear G'_{ST} (G'_{STn})	0.281	0.124
Pollen /seed migration ratio *r*	3.331	31.608

(Carp20 and Carp24) from 264 individuals in 43 populations . Therefore, these three markers (Carp20, Carp21, and Carp24) were excluded from further genetic analyses. Consequently, 11 nuclear microsatellite markers were used for genetic analyses.

Characteristics of the microsatellite loci of Magnolia obovata *and* Carpinus laxiflora

In *Magnolia obovata*, only one marker (M6D10) showed significant deviation from HWE in one (No. 40) of the eight populations examined ($P < 0.05$ after Bonferroni correction). Significant LD was not detected for any pairs of loci in any populations. No outlier loci were detected with excessively high or low F_{ST} values compared with the neutral expectation. Based on these results, all seven loci were used for further analyses.

In *Magnolia obovata, AR* ranged from 3.94 to 5.5, *PA* from 0 to 0.24, H_O from 0.667 to 0.833, and H_E from 0.7 to 0.84 (Appendix 6). The mean values of *AR, PA,* H_O, and H_E were 4.87, 0.10, 0.71, and 0.71, respectively.

In *Carpinus laxiflora, AR* ranged from 3.94 to 5.5, *PA* from 0 to 0.31, H_O from 0.6 to 0.795, and H_E from 0.634 to 0.773 (Appendix 7). The mean values of *AR, PA,* H_O, and H_E were 5.59, 0.18, 0.76, and 0.78, respectively. No outlier loci were detected with excessively high or low F_{ST} values compared with the neutral expectation.

The overall genetic differentiation among populations at the 7 and 11 loci for *Magnolia obovata* and *Carpinus laxiflora*, respectively, was low (G_{ST}, G'_{ST} = 0.044, 0.281 and 0.029, 0.124 among the *M. obovata* and *C. laxiflora* populations, respectively) (Table 1). *Magnolia obovata* had higher G'_{ST} values than *C. laxiflora*.

Genetic structure estimated using nuclear microsatellite markers

In both species, the value for ΔK was the highest when $K = 2$ (Appendix 8). Therefore, we considered the appropriate number of clusters (K) to be two. The assignment probabilities into two clusters for each individual are shown in Fig. 4 with information from the cpDNA haplotypes (Tono *et al.* 2015). Possible admixing individuals with nearly the same levels of probabilities of cluster I and II were observed in several populations of the two species.

Geographic distributions of the STRUCTURE clusters in the two species are shown in Fig. 2, together with those of the cpDNA haplotypes. In *Magnolia obovata*, the proportion of cluster I (in blue) was high in populations from the eastern and Sea of Japan side of the Kinki-Chugoku region, whereas the proportion of cluster II (in red) was high in the populations from the western and Pacific Ocean side of the region. In *C. laxiflora*, the proportion of cluster I (in blue) was high in populations from the eastern part of the Kinki-Chugoku region, whereas the proportion of cluster II (in red) was high in populations from the western part of the region.

Pollen/seed migration ratio

The overall genetic differentiation among populations (G'_{ST}) at cpDNA of *Magnolia obovata* and *Carpinus laxiflora* was 0.675 and 0.827, respectively. The pollen/seed migration ratio *r* of *M. obovata* and *C. laxiflora* estimated on the basis of the values of G'_{ST} was 3.33 and 31.60, respectively (Table 1).

Discussion

Two nuclear genetic clusters were detected in both species by STRUCTURE analysis. In *Magnolia obovata*, the results based on nuclear microsatellites and cpDNA haplotypes were highly consistent (Fig. 2). In contrast, in *Carpinus laxiflora*, the genetic structure based on nuclear microsatellites and cpDNA haplotypes was not as concordant as in *M. obovata* (Fig. 2). Moreover, in *M. obovata*, the difference in frequencies in clusters 1 and 2 was observed not only between the eastern and western parts but also between the northern (Sea of Japan side) and southern parts of the Kinki-Chugoku region. In previous phylogeographic studies of *M. obovata* (Iwasaki *et al*. 2012), three major cpDNA haplotypes with different main distribution ranges were found: haplotype A in eastern Japan, B in southwestern Japan, and C in the Sea of Japan side of Honshu. They concluded that the genetic structure could have been shaped by isolation of the populations into several different refugia in each of the areas during the last glacial period. In this study, individuals with the nuclear cluster I (blue) and cpDNA haplotype C and those with the nuclear cluster II (red) and cpDNA haplotype B were frequently observed in the Sea of Japan side area and western Japan, respectively. Therefore, the northeast-southwest genetic divergence of the nrDNA observed in the Kinki-Chugoku region in this study may be mainly attributed to historical isolation of the populations between the Sea of Japan side refugia and the southwestern Japan refugia during LGM. However, further studies with wider sampling are necessary to reveal the migration history that shaped the northeast-southwest differentiation in this region.

Maternally inherited cpDNA can only be dispersed through seeds, whereas bi-parentally inherited nrDNA can be dispersed through seeds and pollen. Therefore, if geographical distribution patterns for nrDNA clusters and cpDNA haplotypes are consistent, it means that dispersability through seeds and through pollen differs little. Additionally, the pollen/seed migration ratio in *Carpinus laxiflora* (r = 31.6) was much higher than in *Mgnolia obovata* (r = 3.33) (Table 1). At the same time, the geographic distribution patterns of cpDNA haplotypes in the two species reported by Tono *et al.* (2015) were very similar, suggesting that migration rates through seeds differ little between the two species. The large differences in nuclear genetic structure as well as in the values of the pollen/seed migration ratios between the two species likely reflect differences in the amount of gene flow through pollen.

Dissolution of the genetic structure after the LGM may be related to differences in dispersability through pollen and seeds. Hamrick *et al.* (1990) indicated that wind-pollinated species have a higher rate of gene flow than do animal-pollinated species based on differences in G_{ST} values between wind- and animal-pollinated species. For example, wind-pollinated *Fagus crenata* Blume was reported to show marked genetic structure in a molecular phylogeographic analysis based on cpDNA markers (Fujii *et al.* 2002). However, the genetic structure based on nrDNA SSR markers was not as clear as studies based on cpDNA markers (Hiraoka & Tomaru 2009). Similarly, in wind-pollinated *Betula maximowiczziana* Regel, *Picea jezoensis* Maxim., *Cercidiphyllum japonicum* Siebold & Zucc., *Juglans mandshurica*, and *J. cathayensis*, the genetic structures observed on the basis of nrDNA SSR markers were also not as clear as those based on cpDNA markers (Tsuda & Ide 2005, 2010, Aizawa *et al.* 2007, 2009, Bai *et al.* 2010, 2014, Qi *et al*., 2012). In contrast, insect-pollinated *Melampodium leucanthum* Torr. & A. Gray, *Sinopodophyllum hexandrum* (Royle) T. S. Ying, *Cyananthus delavayi* Franch., *Kalopanax septemlobus*, *Shorea leprosula*, and *Callicarpa japonica* Thunb., showed similar and marked genetic structure based on both nrDNA and cpDNA markers (Rebernig *et al.* 2010, Li *et al.* 2011, 2012, Sakaguchi *et al.* 2012, Ohtani *et al.* 2013, Hirano *et al.* 2014).

The results concordantly suggest that differences between the genetic structures revealed by cpDNA and nrDNA markers may be caused by the movement of pollen, which is greater than

movement of seeds in wind-pollinated species. The wind-pollinated *Carpinus laxiflora* plausibly has a higher migration rate through pollens dispersal than in the insect-pollinated *Magnolia obovata*. Accordingly, differences in nrDNA genetic structure between *M. obovata* and *C. laxiflora* is likely due to difference in the mode of dispersal of pollen.

Results from this study help to clarify the influence of life history traits, such as mode of pollen dispersal on dissolution of genetic structure. Hamrick *et al.* (1990) reported that the mode of pollen dispersal has a large influence on the genetic structure of plants. By considering phylogenetic constraints, Duminil *et al.* (2007) suggested that differences in the mode of pollen dispersal do not affect genetic differentiation patterns. These suggestions by two previous studies are important but contradictory. Several reasons can be postulated for this contradiction. Duminil *et al.* (2007) did not consider the effects of differences in the migration histories in their meta-analyses, although many phylogeographic studies have reported different spatial genetic structures and different migration histories for various species. Furthermore, their studies have included various geographic study systems whose topographic factors and geographic scales are different. Such differences may influence migration histories and patterns of genetic differentiation. Thus, comparative studies focused on co-distributed species with similar migration histories in the same geographic research system, such as contact zones as in the present study, will provide valuable suggestions about relationships between life historical traits and population genetic structure. This study demonstrates that contact zones can be good systems for examining the effects of life history traits, such as pollen dispersability, on genetic structure.

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APPENDIX 1. Location of the sampling sites and chloroplast DNA haplotype information for each population.

No	Locality	Altitude (m)	Collector	Coordinates	*Magnolia obovata*			*Carpinus laxiflora*			
					Number of samples	Haplotypes East	Haplotypes West	Number of samples	Haplotypes East	Haplotypes West	
1	Japan, Shiga Pref., Yogo, Mt. Yokoyamadake	1058	T. Iwasaki	35°36′N, 136°15′E	2	1	1				Tono *et al.* 2015, Original
2	Japan, Shiga Pref., Yogo, Mt. Gyoiti	296	A. Tono	35°34′N, 136°11′E	6	6		12	11	1	Tono *et al.* 2015, Original
3	Japan, Shiga Pref., Yogo, Japan National Route 365	637	A. Tono	35°42′N, 136°09′E	6	5	1	15	15		Tono *et al.* 2015, Original
4	Japan, Fukui Pref., Tsuruga, Kuroga forest road	243	A. Tono	35°34′N, 136°03′E				15	15		Tono *et al.* 2015, Original
5	Japan, Shiga Pref., Takashima, Mt. Akasaka	539	A. Tono	35°27′N, 135°59′E				8	7	1	Tono *et al.* 2015
6	Japan, Fukui Pref., Kohama, Onyu Pass North	434	A. Tono	35°24′N, 135°48′E				20	16	4	Tono *et al.* 2015, Original
7	Japan, Fukui Pref., Ooi, Gonami Pass	413	A. Tono	35°21′N, 135°42′E				19	9	10	Tono *et al.* 2015, Original
8	Japan, Kyoto Pref., Kyoto, Kita-ku, Kyotanba, Mt.Chourougatake	555	A. Tono	35°19′N, 135°27′E				6	2	4	Tono *et al.* 2015
9	Japan, Kyoto Pref., Ayabe, Mt Misen	503	A. Tono	35°24′N, 135°22′E				6	6		Tono *et al.* 2015
10	Japan, Kyoto Pref., Yasaka, Mt. Taiko	659	T. Iwasaki	35°41′N, 135°12′E	3	3					Tono *et al.* 2015
11	Japan, Kyoto Pref., Fukuchiyama, Mt Shiro	277	A. Tono	35°26′N, 135°08′E				6	6		Tono *et al.* 2015
12	Japan, Kyoto Pref., Fukuchiyama, Mt. Ooe	817	T. Iwasaki	35°27′N, 135°6′E	4	3	1				Tono *et al.* 2015, Original
13	Japan, Hyogo Pref., Toyooka, Toubi Pass	379	A. Tono	35°25′N, 135°02′E				8	5	3	Tono *et al.* 2015
14	Japan, Hyogo Pref., Tanba, Enoki Pass	317	A. Tono	35°16′N, 135°01′E	1		1	3	0	3	Tono *et al.* 2015
15	Japan, Hyogo Pref., Toyooka, Mt.Nishitokonoosan	609	A. Tono	35°25′N, 134°55′E	16	11	5	6	5	1	Tono *et al.* 2015, Original
16	Japan, Hyogo Pref., Asago, Mt. Awaga	530	A. Tono	35°13′N, 134°53′E				1	1		Tono *et al.* 2015
17	Japan, Hyogo Pref., Kamikawa, Japan National Route 367	445	A. Tono	35°07′N, 134°50′E				1	1		Tono *et al.* 2015
18	Japan, Hyogo Pref., Kamikawa, Mt. Kasagata	366	A. Tono	35°04′N, 134°49′E	4	1	3				Tono *et al.* 2015
19	Japan, Hyogo Pref., Asago, Touwa Pass	542	A. Tono	35°19′N, 134°47′E	2	1	1	3	1	2	Tono *et al.* 2015
20	Japan, Hyogo Pref., Asago, Mt. Tarugamine	451	A. Tono	35°10′N, 134°46′E	1		1				Tono *et al.* 2015
21	Japan, Hyogo Pref., Yabu, Mt. Myouken	725	A. Tono	35°25′N, 134°39′E	3	1	2				Tono *et al.* 2015
22	Japan, Hyogo Pref., Himeji, Mt. Seppiko	560	A. Tono	35°04′N, 134°38′E				1	1		Tono *et al.* 2015
23	Japan, Hyogo Pref., Kami, Mt. Sobu	786	A. Tono	34°13′N, 135°59′E	8	4	4	3	2	1	Tono *et al.* 2015
24	Japan, Hyogo Pref., Shiso, Kouya Pass	702	A. Tono	35°11′N, 134°35′E	3	1	2	1		1	Tono *et al.* 2015
25	Japan, Hyogo Pref., Ichinomiya, Mt. Hujinashi	923	T. Iwasaki	35°16′N, 134°35′E	2	1	1				Tono *et al.* 2015
26	Japan, Hyogo Pref., Yabu, Mt. Hyouno	1048	A. Tono	35°21′N, 134°32′E	18	8	10	6	3	3	Tono *et al.* 2015, Original
27	Japan, Hyogo Pref., Shiso, Mt. Kuroo	539	A. Tono	35°06′N, 134°31′E				1	1		Tono *et al.* 2015
28	Japan, Hyogo Pref., Shiso, Akatani valley	486	A. Tono	35°13′N, 134°30′E				2		2	Tono *et al.* 2015
29	Japan, Hyogo Pref., Kami, Mt. Senno	988	A. Tono	35°27′N, 134°27′E	7	6	1	3	3		Tono *et al.* 2015
30	Japan, Tottori Pref., Yazu, Mt. Okino	793	A. Tono	35°16′N, 134°20′E	12	7	5	6	6		Tono *et al.* 2015
31	Japan, Okayama Pref., Bizen, Mt. Hattoji	257	A. Tono	34°55′N, 134°16′E				1		1	Tono *et al.* 2015
32	Japan, Tottori Pref., Yazu, Mt. Nagi	800	A. Tono	35°11′N, 134°11'				8	6	2	Tono *et al.* 2015
33	Japan, Tottori Pref., Tottori, Japan National Route 118	471	A. Tono	35°18′N, 134°07′E	1	1					Tono *et al.* 2015
34	Japan, Okayama Pref., Tsuyama, Okutusgawa valley	427	A. Tono	35°13′N, 134°01′E				1		1	Tono *et al.* 2015
35	Japan, Tottori Pref., Tottori, Tatsumi Pass	561	A. Tono	35°19′N, 134°01′E	3	1	2	5	5		Tono *et al.* 2015
36	Japan, Okayama Pref., Kagamino, Mt. Izumi	551	A. Tono	35°13′N, 133°59′E	7		7	7		7	Tono *et al.* 2015
37	Japan, Okayama Pref., Kume, Mt. Tenshi	649	A. Tono	34°59′N, 133°53′E	2		2	6		6	Tono *et al.* 2015
38	Japan, Okayama Pref., Tsuyama, Mt. Genjyuji	468	A. Tono	35°03′N, 133°52′E	8		8				Tono *et al.* 2015, Original
39	Japan, Okayama Pref., Kagamino, Mt. Oozora	739	A. Tono	35°12′N, 133°50′E	4	2	2	6	1	5	Tono *et al.* 2015
40	Japan, Okayama Pref., Maniwa, Mt. Tsuguro	859	A. Tono	34°15′N,133°47°E	12	7	5				Tono *et al.* 2015, Original
41	Japan, Okayama Pref., Maniwa, Oohira Pass	458	A. Tono	35°10′N, 133°45′E	5	1	4				Tono *et al.* 2015
42	Japan, Okayama Pref., Kibichuo Mt. Yamato	608	A. Tono	34°49′N, 133°41′E				9		9	Tono *et al.* 2015
43	Japan, Tottori Pref., Kouhu, Mt. Karasuga	1206	T. Iwasaki	35°21′N, 133°34′E	4	3	1				Tono *et al.* 2015
44	Japan, Okayama Pref., Shinjyo, Mt. Kenashi	1204	T. Iwasaki	35°14′N, 133°30′E	6	2	4				Tono *et al.* 2015, Original
45	Japan, Okayama Pref., Takahashi Mt. Tenjin	681	A. Tono	34°52′N, 133°25′E	1		1	5		5	Tono *et al.* 2015
46	Japan, Okayama Pref., Niimi, Mt. Hanami	1005	A. Tono	35°09′N, 133°24′E				1		1	Tono *et al.* 2015
47	Japan, Tottori Pref., Houki, Mt. Kamakura	594	A. Tono	35°15′N, 133°23′E	5	2	3	14		14	Tono *et al.* 2015
48	Japan, Hiroshima Pref., Jinsekikogen, Japan National Route 9	584	A. Tono	34°45′N, 133°22′E	1		1	8		8	Tono *et al.* 2015, Original
49	Japan, Okayama Pref., Takahashi, Japan National Route 313	553	A. Tono	34°52′N, 133°21′E	2		2				Tono *et al.* 2015
50	Japan, Hiroshima Pref., Shobara Mt. Dougo	1204	A. Tono	35°04′N, 133°14′E	7		7	5		5	Tono *et al.* 2015, Original
51	Japan, Hiroshima Pref., Sanwa, Mt. Hoshinoko	719	T. Iwasaki	34°44′N, 133°13′E	1		1				Tono *et al.* 2015
52	Japan, Hiroshima Pref., Fuchuu, Mt. Take	378	A. Tono	34°38′N, 133°10′E	9		9				Tono *et al.* 2015, Original
53	Japan, Hiroshima Pref., Shobara Mt. Takashiburo	383	A. Tono	34°48′N, 133°08′E				2		2	Tono *et al.* 2015, Original
54	Japan, Hiroshima Pref., Miyoshi, Mt. Kagenobu	453	A. Tono	34°45′N, 133°05′E				1		1	Tono *et al.* 2015, Original
55	Japan, Shimane Pref., Unnan Mt. Tengu	465	A. Tono	35°19′N, 133°04′E	1		1	8		8	Tono *et al.* 2015
56	Japan, Shimane Pref., Okuizumo, Mt. Azuma	727	A. Tono	35°05′N, 133°02′E	1		1				Tono *et al.* 2015
57	Japan, Hiroshima Pref., Shobara Mt. Himuro	714	A. Tono	35°00′N, 132°54′E	7		7	6		6	Tono *et al.* 2015
58	Japan, Hiroshima Pref., Takano, Mt. Ooyorogi	926	T. Iwasaki	35°6′N, 132°52′E	10		10				Tono *et al.* 2015
59	Japan, Hiroshima Pref., Akitakata, Mt. Oojichiyama	539	A. Tono	34°39′N, 132°46′E	12		12	8		8	Tono *et al.* 2015, Original
60	Japan, Hiroshima Pref., Higashihiroshima, Mt. Noro	714	A. Tono	34°31′N, 132°43′E	11		11	7		7	Tono *et al.* 2015, Original
61	Japan, Shimane Pref., Oota, Mt. Sanbe	560	A. Tono	35°09′N, 132°38′E				4		4	Tono *et al.* 2015, Original
62	Japan, Shimane Pref., Onan, Mt. Kan	410	A. Tono	34°56′N, 132°31′E	8		8				Tono *et al.* 2015, Original
63	Japan, Shimane Pref., Misato, Japan National Route 186	258	A. Tono	35°05′N, 132°30′E	2	1	1				Tono *et al.* 2015
64	Japan, Hiroshima Pref., Hiroshima, Mt. Kaiken	680	A. Tono	34°37′N, 132°29′E	4		4				Tono *et al.* 2015
65	Japan, Hiroshima Pref., Geihoku, Mt. Garyuu	1081	T. Iwasaki	34°41′N, 132°11′E	3		3				Tono *et al.* 2015, Original
66	Japan, Yamaguchi Pref., Kano, Mt. Nagano	1008	T. Iwasaki	34°16′N, 131°52′E	15		15				Tono *et al.* 2015, Original

APPENDIX 2. Information of the chloroplast DNA noncoding regions examined in this study.

Plant species	Region	Forward primer	Reverse primer	Sequesnc-ing primers	Sequenced length (bp)1	Reference
Magnolia obovata						
	trnH(GUG)-*psbA* intergenic region	ACTGCCTTGATCCACTTGGC	CGAAGCTCCATCTACAAATGG	Reverse	227	Hamilton 1999
	trnL(UAA)-*trnF*(GAA) intergenic region	GGTTCAAGTCCCTCTATCCC	ATTTGAACTGGTGACACGAG	Reverse	358	Taberlet *et al*. 1991
	trnT(UGU)-*trnL*(UAA)5'exon intergenic region	AATCGAATTCTATTCCATTAAA	CAATCGGAAATGATTCTATC	Forward	356	Iwasaki *et al*. 2012
Carpinus laxiflora						
	trnH(GUG)-*psbA* intergenic region	ACTGCCTTGATCCACTTGGC	CGAAGCTCCATCTACAAATGG	Reverse	402	Hamilton 1999
	trnT(UGU)-*trnL*(UAA)5'exon intergenic region	CGATTAATACTCTAAAAAGAATCTAA	GATCTAGTGTTGGTCCAATCAGA	Forward	288	Iwasaki et al. 2012

1Parts of PCR amplified fragments were sequenced.

APPENDIX 3. Characteristics of seven microsatellite loci for *Magnolia obovata*.

Locus	Repeat motif	Primer sequences (5'-3')	T_a (°C)
M6D3	$(CT)_{22}$	Fw-ACATGGATAGTCGTTGGATA	50
		Rv-ACCCCACTGAAGACAAACAT	
M6D4	$(CA)_2(GA)_{15}$	Fw-CACCGTACCCTATCAGAACC	50
		Rv-ATTTTCAGCATCATCAGTTG	
M6D10	$(GA)_{14}$	Fw-AAATTGTCGTCCAACCAGTT	53
		Rv-AAAGCAGCAAACAGGAAGAG	
M10D3	$(GA)_{35}$	Fw-GTCTAGTGAGCCGCAAATGG	53
		Rv-GTGAACAGCTTTCTTGTGAA	
M10D6	$(CT)_{11}$	Fw-CGACGACGAAACTACTAACA	50
		Rv-TTAACTTGAGGTGGAATGAC	
M15D5	$(GA)_{16}$	Fw-GATCGTTGCTGGCTCGC	52
		Rv-GCCGCCTGGATTATGAA	
M17D5	$(GA)_{19}$	Fw-TGCTGCTCGAAGTTCTGAAT	54
		Rv-CGTGCAGTAAATCAGGATGT	

APPENDIX 4. Characteristics of 14 microsatellite loci for *Carpinus laxiflora*.

Locus	GeneBank accession no.	Repeat motif	Primer sequences (5'-3')	T_a (°C)
Carp2	LC012498	$(AT)_{14}$	Fw-CGTCTCCATTAAAGCCAGCC	57
			Rv-ACTCTTGGCTCCATGTCATG	
Carp3	LC012499	$(AG)_9$	Fw-AGTTGCATGTGGACTGAACC	57
			Rv-CCTTCCATAACCGCCAAAGC	
Carp6	LC012500	$(AT)_{11}$	Fw-CATGACACATAGAGAGGAAGC	57
			Rv-CTCTCCCTTCAACCCTCCAC	
Carp11	LC012501	$(AT)_{12}$	Fw-CGCCCATTCTCTCGCATAAG	57
			Rv-AGCATTCCCTAGTTGTGGTG	
Carp13	LC012502	$(AG)_{13}$	Fw-TGTCTCTGTTCCTCACCACC	57
			Rv-CCTTCCAATGTCCAATGGCG	
Carp16	LC012503	$(AT)_{13}$	Fw-TTTGGTGCGGGTTTACAAGG	57
			Rv-GCACATCCAACCCTTACGAAG	
Carp17	LC012504	$(AT)_9$	Fw-CTGCCATCAATTCCTAGGGC	57
			Rv-TCGAAGCTTATTTCCAGGTGC	
Carp20	LC012505	$(AG)_9$	Fw-CTCCGGTAGTTCTGTCCTCC	57
			Rv-TTCTCTCCACAAACGGACCC	
Carp21	LC012506	$(AG)_{10}$	Fw-TTCGGACCTGGTTCTGTCTG	57
			Rv-TGTCCAAACGCGCTACCAC	
Carp24	LC012507	$(AAT)_{10}$	Fw-GGAAAGACCCACCTTGACC	57
			Rv-CTGTCTAAGCCTCCTGAGCG	
Carp26	LC012508	$(AGAT)_6$	Fw-AATCCAATCCCTAGGTGGCC	57
			Rv-AGAGTTTGAGAGTGAGGTTGC	
Carp27	LC012509	$(AAT)_{15}$	Fw-ACCGACCAAGAGAGTGACATC	57
			Rv-CGAAGGTGAGGTGACGAGTG	
Carp34	LC012510	$(AC)_{12}$	Fw-ACGTGGCTATCTCTGAGTCG	57
			Rv-AGGTATGTTCGCAATGCACG	
Carp41	LC012511	$(AT)_{11}T(AG)_{12}$	Fw-TGCCTCGGTTTAAGTTCAAGC	57
			Rv-AGAAGAACATGGCATGCTGTAG	

APPENDIX 5. Results of the primer screening for 14 microsatellite markers for one population of *Carpinus laxiflora*.

Locus	Size range	*N*	N_A	H_O	H_E	F_{IS}
Carp2	324-354	31	13	0.903	0.867	-0.041
Carp3	328-338	31	5	0.323	0.377	0.144
Carp6	189-210	31	14	0.839	0.860	0.025
Carp11	329-349	31	10	0.710	0.815	0.130
Carp13	229-259	31	13	0.774	0.789	0.018
Carp16	172-192	31	10	0.677	0.807	0.161
Carp17	229-244	31	10	0.806	0.853	0.054
Carp20	220-244	31	11	0.613	0.835	0.266
Carp21	262-266	31	3	0.290	0.530	0.452 ***
Carp24	258-288	31	10	0.710	0.807	0.121
Carp26	358-372	31	5	0.710	0.611	-0.162
Carp27	248-287	31	13	0.645	0.867	0.256
Carp34	361-379	31	8	0.742	0.758	0.021
Carp41	198-217	31	11	0.871	0.843	-0.033

N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, fixation index; ***$P < 0.001$.

APPENDIX 6. Population genetic parameters estimated from nuclear microsatellite data for *Magnolia obovata*.

Population No	*N*	*AR*	*PA*	H_O	H_E
2	6	5.36	0.21	0.74	0.76
3	6	5.15	0.00	0.79	0.75
15	16	5.73	0.22	0.77	0.83
23	8	6.00	0.16	0.71	0.81
26	18	5.68	0.24	0.83	0.83
29	7	4.91	0.22	0.76	0.73
30	12	5.67	0.09	0.67	0.82
36	7	6.16	0.12	0.82	0.81
38	8	4.97	0.13	0.73	0.72
40	12	5.29	0.06	0.68	0.77
41	5	6.00	0.16	0.71	0.76
44	6	5.53	0.07	0.83	0.74
47	5	6.00	0.27	0.83	0.75
50	7	5.65	0.33	0.71	0.79
52	9	5.39	0.13	0.83	0.78
57	7	5.51	0.11	0.69	0.77
58	10	5.90	0.19	0.79	0.81
59	12	6.42	0.68	0.80	0.84
60	11	5.78	0.10	0.73	0.82
62	8	4.47	0.06	0.70	0.70
66	15	5.88	0.24	0.75	0.81

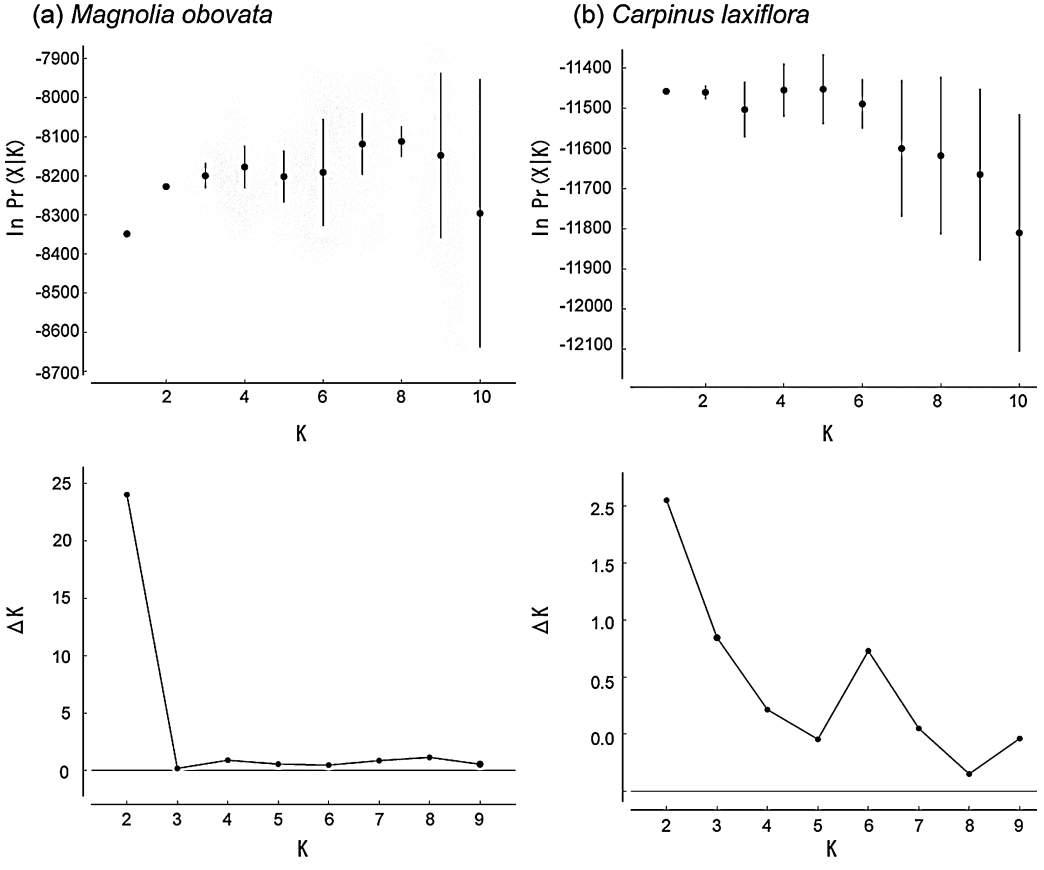
AR, allelic richness; *PA*, private allelic richness; H_E, expected heterozygosity; H_O, observed heterozygosity.

APPENDIX 7. Population genetic parameters estimated from nuclear microsatellite data for *Carpinus laxiflora*.

Population No	*N*	*AR*	*PA*	H_O	H_E
2	12	4.91	0.09	0.67	0.74
3	15	4.85	0.07	0.76	0.74
4	15	4.71	0.02	0.72	0.72
5	8	5.12	0.25	0.70	0.74
6	20	5.26	0.09	0.79	0.77
7	19	5.11	0.04	0.69	0.74
8	6	4.43	0.13	0.74	0.66
9	6	4.42	0.00	0.67	0.68
11	6	4.72	0.00	0.73	0.70
13	8	4.74	0.04	0.69	0.73
15	6	5.42	0.10	0.80	0.74
26	6	5.01	0.09	0.68	0.69
30	6	4.95	0.03	0.71	0.70
32	8	4.76	0.15	0.77	0.69
35	5	5.09	0.13	0.65	0.67
36	7	4.85	0.13	0.64	0.72
37	6	3.94	0.01	0.70	0.63
39	6	4.97	0.03	0.73	0.71
42	9	4.81	0.07	0.69	0.70
45	5	4.91	0.00	0.60	0.69
47	14	5.01	0.12	0.69	0.73
48	8	5.07	0.11	0.80	0.71
50	5	4.73	0.12	0.64	0.64
55	8	5.50	0.31	0.76	0.76
57	6	5.08	0.18	0.73	0.69
59	8	4.66	0.15	0.70	0.69
60	7	4.47	0.31	0.75	0.66

AR, allelic richness; *PA*, private allelic richness; H_E, expected heterozygosity; H_O, observed heterozygosity.

APPENDIX 8. Results of Bayesian clustering (STRUCTURE, Pritchard *et al*. 2000) of *Magnolia obovata* (a) and *Carpinus laxiflora* (b).



The upper graphs show the mean ln Pr (X|K) ± SD over 10 runs for each value of K. The lower graphs show ΔK values for each K (Evanno *et al*. 2005).